

## **Plasma Biomarker Profile Alterations During Variable Blood Storage**

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Running Title: Pre-analytical factors affect blood proteomic profiles

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A major confounding factor in the discovery of disease-specific molecular signatures is the variability in handling clinical blood samples. Consequently, novel protein markers of diseases have failed the “iron test” of validation and implementation into clinical practice because, in addition to patient variability, a range of pre-analytical parameters contributes to erroneous results.

The temperature of storing, transporting and processing of whole blood following collection is one factor that has not been standardized. Often, samples are transferred from the local laboratories to centralised biobanks in a cooled environment or even on dry ice.

Recently, we established a UK biobank to conduct research into the quality of organs donated for transplantation (Quality in Organ Donation, UK QUOD), collecting donor blood samples from 60 UK transplant centres ([www.Quod.org.uk](http://www.Quod.org.uk)). Taking into consideration the logistical challenges in a clinical setting for sample collection and biobanking, we favored whole blood sample handling and processing at ambient temperature ( $22\pm 2^{\circ}\text{C}$ ) prior to isolation of plasma by centrifugation and subsequent storage at  $-80^{\circ}\text{C}$ .

Here, we explored how the proteome and degradome (proteolytic processing of the proteome) may change when whole blood remains at ambient temperature for 30 min, 8 h, 24 h and 48 h prior to plasma preparation. We defined a baseline plasma protein signature that could be used to filter candidate protein markers in future biomarker discovery studies. To this end, 40 ml of blood from five healthy individuals was collected according to our research consent policy. EDTA gel vacutainer plasma gel separator tubes (BD, with Pearlescent White Hemogard Closure) were used without proteinase inhibitors, and blood samples remained at ambient temperature for 30 min, 8 h, 24 h and 48 h prior to centrifugation ( $1500 \times g$  for 15 min at  $22^{\circ}\text{C}$ ) for plasma isolation. No haemolysis was observed in any of the blood samples before or after blood centrifugation or during the period of 48 h at ambient temperature.

Following depletion of the top 14 plasma proteins using a MARS column (Agilent), plasma

proteome signatures of all time points were compared by label-free quantitative mass spectrometry (LFQ LC-MS/MS) (1) and protein degradation profiles were mapped by PROTOMAP (2,3) at 30 min and 48 h. LFQ LC-MS/MS analysis of the in-solution tryptic digests was performed by nano ultra-high performance liquid chromatography-tandem mass spectrometry (nUHPLC-MS/MS) coupled to a Q Exactive tandem mass spectrometer (Thermo Scientific, Bremen, Germany), as previously described (1). Quantification of each protein across all time points was performed by PROGENESIS QI for Proteomics (QIP) software v3.1.4003.30577 (Nonlinear Dynamics) based on the peptide intensity at the MS1 level following chromatogram alignment.

Quantification analysis of all time points indicated that proteins were remarkably stable at ambient temperature up to 48 h with no obvious trend of change with time (at least 2 unique peptides and >95% confidence in protein identification, (1% FDR). Changes in protein abundance occurred in less than 5% of the proteome, and 20 proteins showed significantly increased concentrations over time (ANOVA  $p \leq 0.05$ , Table 1). Amongst these were thrombospondin-1 and cystatin C, both previously defined biomarkers (4,5). The majority of proteins that were enriched with time were of platelet or leucocyte origin, suggesting a low level of cell activation possibly due to sheer stress of blood extraction from the vascular system during collection.

The two extreme conditions, 30 min and 48 h, were selected to further investigate the full effect of proteolysis by PROTOMAP analysis. Five individual samples per condition were pooled to one, proteins were separated by 1-D SDS PAGE, and divided into 22 gel bands per condition. Proteins captured in gel bands were digested and analysed by LC-MS/MS. PROTOMAP bioinformatics combined information from the 1-D gel migration of protein fragments and LC-MS/MS analysis to map the degradation profile for each identified protein (2,3). 820 unique proteins were identified and 22 proteins demonstrated a clear proteolysis profile with protein

fragments identified in lower molecular weight bands in T=48 h when compared to the “parent protein” identified in higher molecular weight bands in T=30 min plasma samples. A high number of the protein fragments detected in T=48 h plasma samples were derived from complement and coagulation factors, indicating partial proteolytic processing upon blood storage possibly due to plasma circulating proteases (Table 1).

In summary, we have produced a reference list of proteins that are significantly altered when whole blood is stored in ambient temperature and demonstrated remarkable few changes in the plasma proteome. This suggests that blood sample storage at ambient temperature maintains proteome integrity for up to 48 h in a logistically challenging environment where samples are donated and collected at multiple hospital sites at any time throughout the day or night.

**Table 1. Pre-analytical effects on plasma proteome and degradome**

	LFQ LC-MS/MS			PROTOMAP analysis
Protein description	Fold change <sup>a</sup>			T= 48h vs T=30m
	T= 8h vs T=30m	T= 24h vs T=30m	T= 48h vs T=30m	
Fibrinogen gamma chain	1.4	1.3	1.2	No Degradation
Apolipoprotein A-I	1.3	1.3	1.3	No Degradation
Profilin-1	1.1	2.0	7.1	No Degradation
DNA polymerase epsilon catalytic subunit A	1.2	1.2	1.2	No Degradation
Coiled-coil domain-containing protein 11	1.2	1.3	1.3	No Degradation
Coagulation factor XIII B chain	1.2	1.2	1.2	No Degradation
Heat shock protein 70 kDa	1.3	1.3	1.2	No Degradation
Dedicator of cytokinesis protein 7	1.5	1.5	1.4	No Degradation
Cystatin-C	1.3	1.3	1.3	No Degradation
Uncharacterized protein C2orf53	1.8	1.9	1.7	No Degradation
Complement C1q subcomponent subunit B	1.3	1.3	1.3	No Degradation
Leukocyte immunoglobulin-like receptor	1.2	1.4	1.4	No Degradation
MCM domain-containing protein 2	1.6	1.7	1.7	No Degradation
Ankyrin repeat domain-containing protein 54	2.2	2.3	2.2	No Degradation
Thrombospondin-1 <sup>b</sup>	1.2	1.3	1.4	Partially degraded
Coagulation factor XI <sup>c</sup>		N/E		Partially degraded
Complement C1r <sup>c</sup>		N/E		Partially degraded
Actin <sup>c</sup>		N/E		Partially degraded
Complement C3 <sup>c</sup>		N/E		Partially degraded
Complement C4B <sup>c</sup>		N/E		Partially degraded
Talin-1 <sup>c</sup>		N/E		Partially degraded
Apolipoprotein B-100 <sup>c</sup>		N/E		Partially degraded
Complement C5 <sup>c</sup>		N/E		Partially degraded
Ceruloplasmin <sup>c</sup>		N/E		Partially degraded
Alpha-1 antichymotrypsin <sup>c</sup>		N/E		Partially degraded
Inter alpha trypsin inhibitor <sup>c</sup>		N/E		Partially degraded
Kallistratin <sup>c</sup>		N/E		Partially degraded
Corticosteroid-binding globulin <sup>c</sup>		N/E		Partially degraded
Serum paraoxonase <sup>c</sup>		N/E		Partially degraded
Fibronectin <sup>c</sup>		N/E		Partially degraded
Plasmonogen like protein A <sup>c</sup>		N/E		Partially degraded
Complement C2 <sup>c</sup>		N/E		Partially degraded
Fibrinogen alpha chain <sup>c</sup>		N/E		Partially degraded
Extracellular matrix protein-1 <sup>c</sup>		N/E		Partially degraded
Collagen alpha 3 <sup>c</sup>		N/E		Partially degraded
Protein disulphide isomerase A3 <sup>c</sup>		N/E		Partially degraded

<sup>a</sup> The shaded part of the table demonstrates proteins that were identified and quantified by PROGENESIS QI and were compared across all time points (ANOVA  $P \leq 0.05$ ). A selection of the identified proteins is included.

N/E indicates that the abundance of these proteins was not changed significantly as measured by LFQ LC-MS/MS, but fragments from these proteins were identified in the lower molecular weight range by PROTOMAP analysis.

<sup>b</sup> Thrombospondin was markedly increased between T=30 min and later time points in addition to the generation of proteolytic fragments observed in T=48h samples.

<sup>c</sup> Indicates PROTOMAP results. All MS data has been deposited to PRIDE repository (PXD004205 and 10.6019/PXD004205).

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The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004205 and 10.6019/PXD004205

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